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ABSTRACT

The protein that is defective in patients with neurofibromatosis type 1 (NF1) functions as a GTPase Activating Protein (GAP) for Ras, yet homozygous loss of a highly conserved Drosophila NF1 ortholog results in several phenotypes that are insensitive to manipulating Ras signal transduction, but rescued by increasing signaling through the cyclic AMP-dependent protein kinase A (cAMP/PKA) pathway. To study how NF1 modulates this pathway and to evaluate whether the cAMP/PKA pathway represents a valid therapeutic target in NF1, the aims of this project are to perform a comprehensive structure-function transgenic analysis to assess the *in vivo* importance of protein domains. We also aim to investigate whether different phenotypes reflect roles for NF1 as a GAP for either conventional Ras ortholog Ras1 or for R-Ras ortholog Ras2, to determine whether a neuroendocrine defect explains the non-autonomous *Nf1* growth deficiency, and to perform other experiments to determine why growth is restored by increasing PKA activity. Finally, with al long term goal of manipulating human NF1 expression levels, we aim to identify cis-acting elements and their cognate transcription factors that drive Drosophila *Nf1* expression in larval CNS neurons and in peripheral nerves.

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Body

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1.4.3 Generate *Nf1* promoter – GFP reporter constructs.

The following describes progress made towards achieving these goals.

1.1.1 Generate transgenics for two smaller N-terminal and one smaller C-terminal deletion mutants.

In our original proposal we described that large segments of Drosophila neurofibromin are dispensable for rescuing the *Nf1* pupal size phenotype. Thus, neurofibroma mutants that carry in-frame deletions of amino acids 492-1094 upstream of and including part of the N-IRA homologous region, of amino acids 1611-1769 representing the Sec14 domain, or of residues 1770-2265 in the C-IRA domain efficiently rescued the size defect when expressed in *Nf1* mutants. By contrast, a transgene that lacked the GAP catalytic domain (amino acids 1219-1580) did not rescue, whereas a transgene representing just the GAP domain was sufficient for rescue. These and other observations support our conclusion that a functional GAP domain is necessary and sufficient for size rescue. However, we also observed that proteins that lacked residues 12-493 or that were truncated beyond amino acid 2347 were expressed, but failed to rescue. Since the GAP domain by itself is sufficient for rescue, we hypothesized that both N and C-terminal segments may control the proper localization of neurofibromin, regulate its stability, or its GAP activity. We noted that the 12-493 segment consists of two evolutionary conserved regions separated by a non-conserved spacer, and proposed to generate two smaller N-terminal deletions that individually removed the conserved segments. To further analyze the importance of these individual conserved domains, we generated transgenes that lack residues 12-284 or 191-492. The rescuing potential of these transgenes has not yet been established. The C-terminal truncation mutant lacks an evolutionary conserved segment that in human neurofibromin interacts with the cytoplasmic segment of syndecans (Hsueh et al., 2001). Although in a directed two-hybrid experiment Drosophila neurofibromin and syndecan showed no interaction, we generated a Drosophila *NF1* transgene that lacks residues 2672 to 2750, corresponding to the C-terminal syndecan binding site in human neurofibromin. A single transgenic line expressing this mutant was obtained, and the transgene crossed into the *Nf1* mutant background.

Interestingly, this 78 amino acid deletion mutant is expressed, but fails to rescue the *Nf1* size defect. To confirm that deletion of residues 2672-2750 results in a functionally deficient protein, we recently generated 10 more transgenic lines. The transgene integration sites of these lines are being mapped, after which several lines with transgenes on the second chromosome will be used to confirm that the 2672-2750 segment is essential for *in vivo* size rescue.

Immunoblot analysis of 3rd instar larval CNS or adult fly head extracts detected elevated levels of MEK and ERK phosphorylation in *NF1* mutants compared to wild-type controls. Thus, we routinely test whether transgenic size rescue correlates with a reduction in P-ERK level. So far we have found a perfect correlation between the two phenotypes. However, it will be interesting to analyze whether the 78 amino acid C-terminal deletion mutant, which has an intact GAP domain but fails to rescue, affects P-ERK levels. If it did not, this would suggest an important role for the C-terminus of neurofibromin in regulating its localization and/or GAP activity. If the mutant did reduce P-ERK levels, this would argue that ERK regulation may not be directly involved in NF1 size control. To further analyze the importance of inappropriate ERK activation in *NF1* growth control, we crossed several *Ras1*, *MEK*, and *ERK* null alleles into the *NF1* mutant background. None of these mutants affects *NF1* pupal size and we are in the process of testing whether they affect larval CNS P-ERK levels. We also obtained a transgenic line expressing a *UAS-MKP3* construct. MKP3 is a Drosophila phosphatase that interacts with and has high specificity for the Drosophila *rolled* ERK ortholog (Kim et al., 2002). We are in the process of analyzing whether ubiquitous or neuronal expression of *UAS-MKP3* down-modulates P-ERK and modifies *NF1* pupal size. Finally, we tested whether mutations in other Ras signaling components, such as PI3-kinase, Ral, and RalGDS, modified *NF1* pupal size. None of these mutants modified the *NF1* size defect.

1.1.2 Generate transgenics for proteasomal degradation motif mutant.

The group of Dr. Karen Cichowski previously reported that human neurofibromin undergoes rapid proteasomal degradation when quiescent cells are stimulated by serum or by various growth factors (Cichowski et al., 2003). Her group identified a conserved KYFTLF motif (residues 1095-1100 in human neurofibromin), which is required for serum-induced degradation. When expressed in mammalian cells, a Drosophila neurofibromin segment that includes this motif showed similar serum-stimulated degradation. Thus, to determine whether proteasomal degradation of neurofibromin is important in vivo, we generated a substitution mutant in which the FTLF residues in the degradation motif (Drosophila NF1 residues 1145-1149) were altered into four alanines. Three lines expressing this transgene were generated and crossed into an *Nf1* mutant background. In all cases the mutant protein efficiently rescued the *Nf1* size defect, arguing that the FTLF box is not essential for function. We also tested whether heat shock induced expression of the mutant protein in a wild-type background affected pupal size, or resulted in any obvious phenotypes. No obvious defects were noted. Thus, proteasomal degradation may regulate the activity of neurofibromin, but a mutant that lacks the identified proteasomal degradation motif appears fully functional and does not cause obvious dominant phenotypes upon overexpression. It remains possible that this mutant will show subtle defects when tested in other assays.

1.1.3 Generate transgenics expressing N-terminal (~1-900) protein segment.

We speculated in our original proposal that N-terminal or C-terminal segments of neurofibromin might interfere with the proper localization or regulation of the protein when overexpressed. We had generated five transgenic lines expressing a C-terminal 455 amino acid segment, and had proposed to similarly generate a transgene expressing residues 1-920 followed by a HA-tag. The idea was to determine whether these transgenes acted in a dominant negative manner when expressed in wild-type flies. We have since analyzed flies expressing the C455 fragment and found no obvious effects on wing or male pupal size. In one line there was a slight but statistically significant reduction in female pupal size, but this effect was small and not observed in smaller male pupae. Routine cloning problems have so far prevented the generation of the 1-920-HA transgene.

1.1.4 Generate full length construct for ERK phosphorylation site mutant.

Serum or growth factor stimulation of several mammalian cell types induces the rapid proteasomal degradation of neurofibromin, followed by its reappearance 30 minutes later (Cichowski et al., 2003). When neurofibromin reappears, it migrates more slowly, which is believed to reflect ERK-mediated phosphorylation. An interesting hypothesis is that ERK phosphorylation activates the GAP activity or stabilizes neurofibromin in the continued presence of growth factors. The only evolutionary conserved, canonical PXS/TP ERK phosphorylation site is located near the C-terminus of human and Drosophila neurofibromin, and preliminary evidence from Dr. Cichowski's lab suggested that this site is indeed phosphorylated by ERK. To test the relevance of this site we changed serine 2741 in the Drosophila NF1 PPSP motif into an alanine and generated a full length transgene. Although not proposed in our original application, we also generated full length transgenes in which serine 2741 was altered into phospho-mimicking aspartate or glutamate residues. As usual, transgenes were extensively mapped and relevant segments sequenced to confirm mutations and guard against unexpected changes. Six transgenic lines harboring the heat-shock inducible S2741A mutant were generated, and none rescued the size defect when induced in a *Nf1* mutant background. However, in immunoblots no NF1 protein expression was detected in any of these six lines. Transgenic lines for the phospho-mimicking mutants have been obtained, but not yet analyzed. We are currently resequencing the injected S2741A transgene and we are exploring whether the S1741A change affects protein stability.

1.1.5 Generate human *NF1-GRD-HA* and *NF1-GRD^{R1276P}-HA* transgenics.

In our hands a Drosophila *NF1-GRD-HA* transgene that includes only the GAP catalytic domain rescued the mutant size defect, whereas two similar transgenes harboring missense mutants that affect GAP activity did not rescue. We obtained similar results with wild-type and GAP deficient full length *NF1* transgenes. These results underlie our conclusion that GAP activity is necessary to rescue the cAMP-sensitive mutant size defect. Yi Zhong's group at Cold Spring harbor Laboratories, however, continues to argue that GAP activity is not essential for size rescue, since in their experiments GAP deficient human *NF1-GRD* transgenes rescued efficiently. Although we used Drosophila transgenes and Zhong used human constructs, this is obviously a major discrepancy. One possibility is that the human mutants down-modulate Ras by interacting with and sequestering the GTPase. To investigate this further we had proposed to generate our own wild-type and GAP deficient (R1276P) human NF1-GRD transgenes. We did generate both transgenes, but in anti-HA immunoblots detected no expression in several transgenic lines. Thus, to properly compare the reagents used, we recently decided to instead exchange transgenes with the Zhong lab. Beyond ongoing attempts to reproduce Zhong's result and investigate its mechanistic basis, we performed several experiments to further determine the importance of GAP activity in the rescue of *NF1* phenotypes. As indicated in our proposal, neuronal expression of *Gap1*, using the available *Gap1^{EP45}* line, did not rescue the *NF1* size defect. However, in recent experiments expression of a full length transgene representing the Drosophila p120GAP ortholog (termed *vap* for *vacuolar peduncle*) does appear to rescue. If confirmed this would provide strong support for our conclusion that GAP activity is necessary and sufficient for rescue. Beyond confirming that vap rescues, we will also determine whether NF1, Gap1, and vap act as GAPs for the same GTPases. Drosophila Ras1 is the sole ortholog of mammalian conventional H-, K- and N-Ras, while Ras2 is closely related to three mammalian R-Ras paralogs (R-Ras1, R-Ras2/TC21, and R-Ras3/M-Ras). In order to test Ras2 in GAP assays we produced the protein in the baculovirus system, since the GST-Ras2 protein that we had planned to use was entirely insoluble. Similar to results obtained with the human protein, a bacterially produced Drosophila NF1-GRD protein potently stimulates the intrinsic GTPase activity of Ras1 and Ras2 (Figure 1). We recently also generated maltose binding protein (MBP) fusion proteins representing the catalytic domains of Gap1 and vap. We are in the process of testing their in vitro GTPase specificities. We have also created our own full length *UAS-Gap1* transgene. We will generate transgenic lines that express this transgene at various levels, to further confirm that Gap1 is unable to substitute for NF1 in growth regulation.

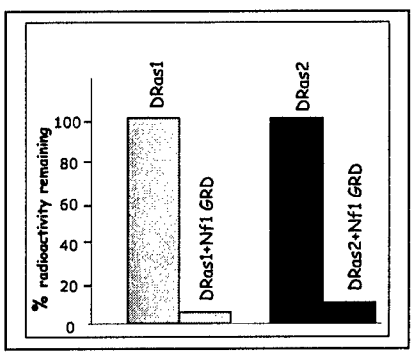

Figure 1. A Drosophila NF1-GRD MBP fusion protein stimulates the GTPase activity of Ras1 and Ras2 in vitro. The graphs show the percentage radioactivity remaining after a 10 minute incubation of 6 nM GTP-γ32P loaded Ras1 or Ras2 in the presence or absence of NF1-GRD protein. No GAP activity was detected in similar assays with Drosophila RalA or Rap1.

1.1.6 Provide characterized transgenics to collaborators for phenotypic rescue analysis.

Beyond exchanging *NF1* transgenes with the lab of Yi Zhong, we have so far provided transgenic flies expressing various *NF1* mutants to collaborators Drs. Scott Waddell (U. Mass) and Michael Stern (Rice U.).

1.1.7 Map and balance new transgenes.

This is routinely performed for any new transgene.

Aim 2

1.2.1 Analyze whether tissue-specific expression of *UAS-Ras1* or *UAS-Ras2* causes defects and/or enhances *Nf1* phenotypes.

We generated transgenics expressing wild-type *UAS-Ras2*, activated *UAS-Ras2^{val14}*, and dominant negative *UAS-Ras2^{N19}*. Transgenic lines expressing the corresponding Ras1 mutants were obtained from the Bloomington Stock Center. We had proposed to test whether over-expression of wild-type Ras1 or Ras2 enhances *Nf1* defects, based on a published report that overexpression of Ras1 enhances the brain degeneration phenotype of the Drosophila vap mutant (Botella et al., 2003). However, this latter result has been called into question (David Hughes, personal communication), and overexpression of either *UAS-Ras1* or *UAS-Ras2* under the control of several GAL4 drivers did not modify the *Nf1* size defect.

1.2.2 Analyze effects of tissue-specifically expressing *UAS-Ras1^{N17}* and *UAS-Ras2^{N19}* in wild-type and *Nf1* backgrounds.

Although not originally proposed, we generated several Drosophila lines that express the yeast GAL4 transcription factor under the control of the *Ras1* or *Ras2* transcriptional promoters. *Ras1-GAL4* drives *UAS-GFP* expression ubiquitously without discernable pattern, presumably reflecting the ubiquitous expression of Ras1. By contrast, *Ras2-GAL4; UAS-GFP* flies express the GFP reporter in highly specific subsets of cells in the ventral ganglion and the midbrain sections of the larval CNS (Figure 2). Several independent *Ras2-GAL4* lines differ in expression level, but not in expression pattern. This pattern faithfully recapitulates the endogeneous Ras2 mRNA expression pattern (Salzberg et al., 1993). Interestingly, *Ras2-GAL4* drives very little if any *UAS-GFP* expression outside of the larval CNS, arguing for a nervous system specific role for this R-Ras ortholog. Expressing the cell death inducing *UAS-reaper* transgene under the control of *UAS-Ras2* caused early lethality, arguing that Ras2 expressing cells are essential for normal development.

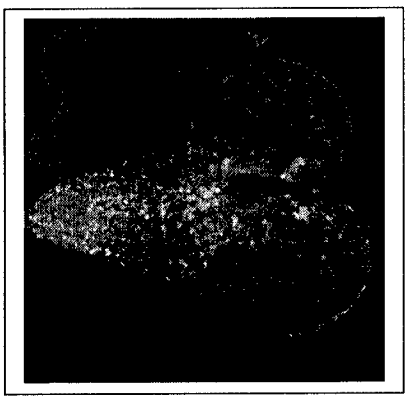

Figure 2; *Ras2-GAL4* driven *UAS-GFP* expression in third instar larval CNS. No obvious UAS-GFP expression is seen in imaginal discs or in any other larval tissue. Driving *UAS-NF1* with *Ras2-GAL4* drivers substantially rescues the *NF1* size defect. Driving activated *Ras1* or *Ras2* mutants with Ras2-GAL4 drivers results in small pupae.

Importantly, expression of *UAS-NF1* under the control of the two strongest *Ras2-GAL4* drivers rescues the *NF1* size defect. Further supporting the idea that *Ras2* expressing cells play an important role

in NF1-mediated growth regulation, expressing activated *UAS-Ras2^{val14}* under the control of *Ras2-GAL4* results in small pupae, which do not eclose. A similar result was obtained when *Ras2-GAL4* is used to drive expression of the activated *UAS-Ras1^{val12}* mutant. We are in the process of determining whether expression of dominant negative *UAS-Ras1^{N17}* or *UAS-Ras2^{N19}* mutants under the control of *Ras2-GAL4* rescues the *Nf1* size defect. Initially we only obtained a single transgenic line harboring the dominant negative *UAS-Ras2^{N19}* mutant. Expressing this mutant under the control of *Ras2-GAL4* did not modify the *Nf1* size defect. However, we recently generated several more transgenic lines that are currently being tested. In parallel we are testing whether *UAS-Ras1^{N17}* modifies pupal size when driven by *Ras2-GAL4*.

1.2.3 Generate transgenics for short-hairpin pWIZ-Ras2 construct.

As proposed, we generated several transgenic lines expressing a *pWIZ-Ras2* gene knockdown construct. However, ubiquitous expression under the control of the *Act5C-GAL4* driver did not cause any obvious phenotype in wild-type flies, and expression in *NF1* mutants did not modify pupal size. Similar results were obtained using specific neuronal GAL4 drivers. However, in control RNA blots ubiquitous expression of *pWIZ-Ras2* did not reduce the level of endogenous *Ras2* mRNA. Although not proposed, we also generated two different *pWIZ-NF1* short-hairpin constructs to test whether their expression would cause dominant small fly phenotype that might be useful in genetic screens. We again tested several lines for each construct, but none showed any NF1-like phenotype. Researchers in adjacent labs have successfully used the pWIZ short hairpin gene knockdown approach to suppress several non-neuronal genes. Thus, we believe that our failure to suppress *Ras2* or *NF1* may a problem with the pWIZ RNAi approach in neuronal cells. For the time being we have abandoned this experiment.

1.2.4 Test whether induction of four *UAS-GRD-HA* transgenes affects wing size, eye development, or overall development/viability.

At the time of proposal submission we had identified four Drosophila RasGAP-related proteins (NF1, Gap1, vap and SynGAP/CG32560). We have since identified a fifth evolutionary conserved potential RasGAP, CG1657, which interestingly also includes a Vps9 putative RabGEF domain. We had proposed to generate hybrid transgenes that included the catalytic domains of Gap1, vap and SynGAP in the context of full length NF1 backbones, as well as UAS-GRD-HA catalytic domain transgenes for the three initially identified non-NF1 RasGAPs. Our proposal was to use these transgenes to test for functional redundancy. Preliminary data suggested that hybrid *NF1-Gap1-NF1* and *NF1-vap-NF1* proteins did not rescue. However, while we have confirmed this result for the *NF1-vap-NF1* hybrid, we have since found that the Gap1 hybrid is poorly expressed. Similarly, while the *UAS-NF1-GRD-HA* protein is expressed and rescues *NF1* phenotypes, we have since found that in anti-HA immunoblots of transgenic fly lysates, none of the other three catalytic domains is detectable. Thus, we have changed tactics and focused on expressing full length RasGAPs. We previously used an available GAL4-inducible *GAP1^{EP45}* line to show that Gap1 does not substitute for NF1 in growth regulation. However, since a full length p120GAP ortholog does appear to rescue, we recently generated a full length *UAS-Gap1* transgene to confirm that Gap1 does not rescue. The *Gap1^{EP45}* line was generated through random insertion of an enhancer-promoter containing transposon upstream of the Gap1 gene. We have confirmed that expression of *Gap1^{EP45}* in the eye causes a rough eye phenotype that can be suppressed through heterozygous loss of Ras1, as had been reported (Rorth, 1996). However, additional *UAS-Gap1* lines may express higher transgene levels, allowing a more rigorous test of functional redundancy.

1.2.5 Clone *Ras2* genomic segment in preparation for gene targeting.

Before embarking on the labor intensive process of generating a specific *Ras2* mutant through gene targeting, we decided to first obtain more definitive evidence that Ras2 is the relevant target for NF1 in organismal growth control. Thus, rather than generating a Ras2 genomic clone in preparation for gene targeting, we first generated and analyzed *pWIZ-Ras2* transgenics (see section 1.2.3). Although not originally proposed, we also generated and characterized Ras2 promoter driven GAL4 (*Ras2-GAL4*) transgenics (section 1.2.2). The Ras2-GAL4 drivers are expressed in a highly specific subset of cells in

the larval CNS, and driving *UAS-NF1* in this pattern rescues *NF1* pupal size. However, by crossing two deficiencies that include *Ras2* into an *NF1* background, we found that heterozygous loss of *Ras2* does not modify the *NF1* size defect. Thus, we are not yet convinced that Ras2 is the relevant target for NF1 in growth regulation, and we first plan to perform additional GAP assays comparing the catalytic activities of vap, Gap1, and NF1 against several Drosophila Ras related proteins, before attempting to generate specific Ras2 mutants.

Aim 3

1.3.1 Analyze CNS gene expression profiles of *dnc*^{1} and *Nf1;dnc*^{1} 3rd instar larvae.

Microarray analysis of total RNA from developmentally synchronized, wandering stage 3rd instar wild-type and *NF1* larvae revealed >2-fold changes in the expression of 169 genes. Several of these genes showed highly robust, up to 60-fold expression changes. However, similar experiments with RNA from dissected 3rd instar larval CNS identified only few genes whose expression was altered by at most 4.5-fold. For many genes these changes in expression were confirmed either by RNA blot or real time RT-PCR analysis. Among the genes that are misexpressed in *NF1* CNS is dopa decarboxylase (*ddc*), whose expression is reduced 4.5-fold, the gene encoding the adipokinetic hormone precursor (*akh*), whose expression is down 3.8-fold, and the neuropeptide-like CG3441/*Nrlp1* gene, whose expression is increased 2-fold. To begin to dissect the mechanism underlying the non-cell-autonomous *NF1* growth defect, we had proposed to analyze which CNS genes are restored to wild-type expression in genetically rescued flies. Specifically, we proposed to compare CNS expression profiles of *dnc*^{1} and *NF1;dnc*^{1} wandering stage 3rd instar larvae. Underlying this experiment was our observation, confirmed by others (Williams et al., 2001), that loss of the *dnc* cAMP phosphodiesterase rescued *NF1* pupal size. However, when crossed into our most recent isogenized *NF1*$^{E1/E2}$ mutant background, the *dnc*^{1} allele available in our lab no longer modified pupal size. We believe this reflects a problem with the *dnc*^{1} mutant, since *dnc*^{1} fly extracts showed no increase in cAMP, as had been reported (Byers et al., 1981). A *dnc*^{1} stock subsequently obtained from the Bloomington stock center also behaved abnormally and did not modify *NF1* pupal size. Thus, as described below, we have focused on more direct experiments to reveal the mechanism responsible for the non cell-autonomous NF1 growth deficiency.

1.3.2 Analyze CNS gene expression profile of *dCREB2* hypomorphic 3rd instar larvae.

NF1 and hypomorphic *dCREB2* mutants show similar reductions in postembryonic size, and microarray analysis of whole 3rd instar *NF1* and *dCREB2* larval RNA showed similar changes in gene expression. Thus, since NF1 is required in CNS neurons to rescue overall size, and since only few genes are misexpressed in *NF1* CNS, we proposed to compare the CNS gene expression profiles of developmentally synchronized, wandering stage 3rd instar *NF1, dCREB2* and wild-type larvae. This analysis, performed in triplicate, showed that multiple genes are up or down regulated in *dCREB2* CNS. Thus, while overall 3rd instar larval *NF1* and *dCREB2* gene expression profiles are very similar, only few genes show relatively small expression changes in *NF1* larval CNS, while many more genes show more robust expression changes in *dCREB2* CNS.

Alternatively spliced dCREB2 isoforms function as transcriptional activators and repressors (Yin et al., 1995), which may explain why many genes are either up- or down-regulated in *dCREB2* CNS. The fact that *NF1* and *dCREB2* CNS expression profiles differ significantly is not compatible with the idea that NF1 is a major upstream regulator of adenylyl cyclase/PKA/dCREB2 signals. Further supporting this conclusion, ELISA assays detected identical cAMP levels in extracts of dissected wild type and *NF1* CNS. These results do not rule out that NF1 acts as a regulator of the adenylyl cyclase/PKA/dCREB2 pathway in specific cells in the CNS. However, our results are also compatible with the hypothesis that NF1 alters the expression or secretion of a neuroendocrine factor, which activates the adenylyl cyclase/PKA/dCREB2 pathway in other cells, either in the CNS or elsewhere. Thus, the microarray analysis of dCREB2 CNS has

provided important clues, but have not yet revealed the precise mechanism whereby NF1 causes a cAMP-sensitive overall growth defect.

1.3.3 Induce tissue-specific expression of *UAS-AKH* and analyze *Nf1* size rescue.

Adipokinetic hormone is an eight amino acid hormone that regulates insect energy metabolism in preparation for strenuous activity, such as flight. The *akh* gene codes for the 79 amino acid adipokinetic hormone precursor and is expressed in specific neuroendocrine cells of the Drosophila ring gland (Noyes et al., 1995). Microarray analysis of CNS RNA identified the *akh* gene as being down regulated by approximately 4-fold in *NF1* mutants, which was confirmed by real-time RT-PCR analysis. Intriguingly, the *akh* and *Ras2* genes are immediate genomic neighbors, but *Ras2* expression does not appear to be altered in *NF1* mutants. To analyze whether *NF1* phenotypes are sensitive to increasing *akh* expression, we generated *UAS-akh* transgenics. Induction of this transgene with several neuronal GAL4 drivers, including the *akh-GAL4* driver (see below) did not modify the *NF1* size defect. Thus, adipokinetic hormone does not appear to be involved in the *NF1* size defect.

1.3.4 Generate and characterize *AKH* and *AKH receptor* promoter driven GAL4 lines.

We generated a transgene that has the putative *akh* transcriptional promoter inserted upstream of the *GAL4* coding region. Transgenics harboring this construct were obtained and their transgenes mapped. These flies were subsequently crossed to *UAS-GFP* or *UAS-NF1* transgenics. As expected, the *akh-GAL4* driver induced *UAS-GFP* expression in a very small number of cells in the neuroendocrine larval ring gland (not shown). Driving *UAS-NF1* in this highly specific pattern did not modify *NF1* pupal size. Expressing *UAS-NF1* under the control of several other ring gland drivers also does not affect *NF1* pupal size. *CG3441/Nplp1* is the only neuropeptide gene whose expression is increased (by about 2-fold) in *NF1* CNS. Although not proposed, we generated several *UAS-CG3441* transgenics as well as several lines that harbor a *CG3441* promoter-driven *GAL4* construct. The latter lines drive *UAS-GFP* expression in highly specific cells in the CNS ventral ganglion and the midbrain region (Figure 3). This pattern strongly resembles the endogenous *Nplp1* expression pattern (Verleyen et al., 2004). Recent results indicate that *CG3441-GAL4* driven *UAS-NF1* expression does not alter *NF1* pupal size. However, Drosophila neuroendocrinologist Dr. Paul Taghert has generated *CG3441* hypomorphic mutants and identified a behavioral phenotype. We are interested in determining whether this phenotype resembles the uncoordinated phenotype of *NF1* mutants (The et al., 1997). If these phenotypes are related, it will be interesting to test whether altered *CG3441* expression plays any role. We have started a collaboration with Dr. Taghert to analyze this and other possibilities.

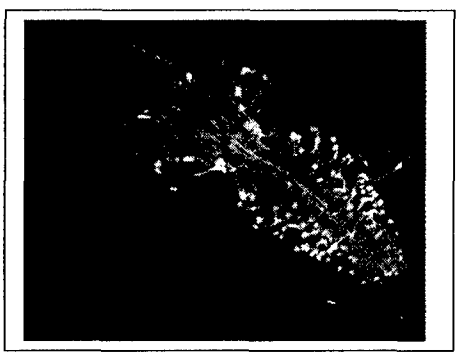

Figure 3; *CG3441-GAL4* driven *UAS-GFP* expression in 3rd instar larval CNS.

All experiments proposed under this aim have the broad goal of identifying the precise mechanism whereby loss of NF1 in the larval CNS causes an overall growth defect. As described in our grant proposal, several of our preliminary results argued against the idea that *NF1* mutants are small because they are nutritionally deprived. Insulin is a major neuroendocrine growth regulator in Drosophila, but several additional results argued that insulin signaling was normal in *NF1* mutants. However, to further analyze a potential role for insulin, we analyzed the life span of *NF1* mutants, given that some mutants with attenuated insulin signaling have a significantly increased life span (Clancy et al., 2001; Tatar et al., 2001). However, the life span of *NF1* mutants and isogenic controls was very similar. Several insulin pathway mutants also develop more slowly. Interestingly, when we analyzed developmental timing, *NF1* mutants were found to undergo significantly accelerated development. We performed this experiment by comparing trans-heterozygotes for two *NF1* EMS alleles ($NF1^{E1}/NF1^{E2}$) to isogenic control flies. In these experiments the *NF1* mutants pupariated at least 10 hours before controls. Wild-type flies undergo a

developmental transition 70 hours after egg laying (Beadle et al., 1938). Thus, Drosophila larvae die when transferred to a sucrose-only diet before this time. When larvae are transferred to sucrose after 70 hrs, they continue to develop and pupariate at a reduced size. Unlike control flies, we found that *NF1* mutants survive and continue to develop when transferred to sucrose as early as 60 hours after egg laying. Thus, by at least two measures *NF1* mutants develop more rapidly. Initially we were very excited about this finding, because most growth in insects occurs during larval development, and because accelerated development appeared to provide a unique explanation for the small size of *NF1* mutants. However, we have since found that accelerated development is a dominant phenotype specific to the $NF1^{E2}$ allele. Thus, neither $NF1^{E1}$ nor the original $NF1^{P1}$ mutant exhibits accelerated development, and the accelerated development of $NF1^{E1}/NF1^{E2}$ trans-heterozygotes is not rescued by expressing an *NF1* transgene. While we remain interested in determining whether accelerated development is a dominant phenotype of the $NF1^{E2}$ early truncation mutant, or is caused by an unrelated second mutation, we no longer believe that accelerated development provides an explanation for the reduced size phenotype of *NF1* mutants.

1.3.5 Characterize *UAS-dCREB2* isoform transgenics.

We had proposed to analyze whether the size defect of *dCREB2* mutants is cell autonomous or non-autonomous. We also proposed analyzing whether *dCREB2* and *NF1* are required in the same or in different cells to rescue phenotypes. For these experiments we made transgenics representing two *dCREB2* isoforms with opposite roles in transcription and olfactory learning. *UAS-dCREB2a* (activator) and *UAS-dCREB2b* (antagonist) constructs were made, and several transgenic lines obtained for each construct. The *UAS-dCREB2a* transgene has been analyzed most extensively so far. This transgene expressed under the control of the widespread *69B-GAL4* driver rescued the lethality of *dCREB2* females. The same driver and transgene combination also partially rescued the pupal size defect of *dCREB2* mutant males (*dCREB2* male pupal size 2.40 mm, rescued to 2.65 mm by *69B-GAL4; UAS-dCREB2a*. Average wild-type male pupal size is 2.86 mm). The *hsp70-GAL4* driver also allowed partial male pupal size rescue in the absence of heat shock induction. By contrast, the *elav-GAL4* neuronal or repo-*GAL4* glial drivers did not rescue. Finally, expression of *UAS-dCREB2a* under the control of two neuronal drivers (*elav-GAL4* and *c23-GAL4*) did not modify the *NF1* size defect. Obvious follow-up experiments to determine whether widespread expression of *UAS-dCREB2* rescues the *NF1* size defect are in progress.

Aim 4

1.4.1 Generate and characterize transgenics containing 17.3 kb genomic *Nf1* transgene.

At the start of this project a technician was hired to perform these experiments. Unfortunately he left the lab to enter medical school nine months later, before generating this transgene.

1.4.2 Provide transgenic line to Michael Stern to test rescue of glial thickening phenotype.

For reasons explained in section 1.4.1, the generation of this transgenic line has been delayed.

1.4.3 Generate *Nf1* promoter – GFP reporter constructs.

We did generate two constructs that contained approximately 50% and 100% of the genomic region between the 5'-end of *NF1* and the 3'-end of the upstream *CG8968* gene, inserted upstream of the EGFP (enhanced green fluorescent protein) coding region. We also generated constructs in which the same putative *NF1* promoter segments were inserted upstream of the GAL4 coding region. Transgenics harboring In which a *UAS-GFP* reporter is expressed under the control of the two *NF1-GAL4* drivers do not recapitulate the highly distinctive endogenous *NF1* expression pattern. The same is true for transgenics harboring the NF1 promoter driven EGFP reporter constructs. Thus, the genomic segment between the 5' end of *NF1* and the 3'-end of *CG8968* does not appear to include all promoter/enhancer elements required for the neuronal specific expression of NF1. Thus, we will renew our efforts to generate the 17.3 kb genomic transgene and determine whether this region includes all regulatory elements

required for endogenous expression. We note that during the past year three transcription factors have been implicated in regulating the expression of mammalian neurofibromin (Zhu et al., 2004; Chong et al., 2005; Yang et al., 2005). We will continue to monitor this literature and analyze whether Drosophila orthologs of mammalian transcription factors play similar roles.

Key Research Accomplishments:

- Identified 78 amino acid evolutionary conserved C-terminal neurofibromin segment that appears essential for size rescue.
- Established that Drosophila NF1 has GAP activity towards Ras1 and Ras2.
- Generated and characterized *Ras1-GAL4* and *Ras2-GAL4* transgenic driver lines.
- Established that *UAS-NF1* expression under the control of the *Ras2-GAL4* driver rescues the *NF1* size defect.
- Established that *Ras2-GAL4* driven expression of activated mutants of either *Ras1* or *Ras2* mimics the *NF1* size defect.
- Established that ablation of Ras2 expressing CNS cells results in small pupae that do not eclose.
- Obtained preliminary evidence that expression of full length *UAS-vap* rescues *NF1* pupal size.
- Generated and characterized *UAS-akh* and *akh-GAL4* transgenic lines.
- Established that manipulating *akh* expression does not modify *NF1* size defect
- Established that expressing *UAS-NF1* in *akh* pattern, or in other ring gland cells, does not modify *NF1* size defect.
- Generated and characterized *UAS-CG3441* and *CG3441-GAL4* lines.
- Established that expressing *UAS-NF1* in *CG3441* pattern does not modify size defect.
- Identified and characterized accelerated development phenotype specific to $NF1^{E2}$ early truncation mutant.
- Identified genes that are misexpressed in dCREB2 larval CNS.
- Generated and characterized transgenic lines expressing wild-type and mutant dCREB2 isoforms.
- Generated and characterized *NF1* promoter region reporter genes

Reportable outcomes

Publications:

None so far.

Abstracts:

Non-cell-autonomous requirement for NF1-GAP activity underlies Drosophila growth defect. Andre Bernards, James Walker, Anna Tchoudakova, Dongyun Wu. 2005. Children's Tumor Foundation Annual Consortium Meeting, Aspen, CO.

Conclusions:

We achieved most aims identified for year 1, and several aims planned for year 2. We continue to work towards at least one major manuscript that shows that the role of NF1 in organismal growth control is non-cell-autonomous, and that defective GAP activity is the proximal cause of this cAMP-sensitive NF1 phenotype. Submission of thus manuscript has been delayed, because our results argue that GAP activity is essential for in vivo rescue, whereas Zhong et al find that GAP activity is not required. Much of our effort in the past few months had been aimed at resolving this important discrepancy.

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